Journal of Reproduction and Development, Vol. 71, No 2, 2025

Original Article

Involvement of nuclear receptor corepressor 2 (NCOR2) in estrogen-induced repression of arcuate *Kiss1* expression in female rats

Marina TAKIZAWA[1)], Sae MIYAZAKI[1)], Hitomi TSUCHIDA[1)], Mayuko NAGAE[1)], Shunsuke SEKI[1)], Masumi HIRABAYASHI[2)], Fumitaka OSAKADA[3)], Naoko INOUE[1)], Hiroko TSUKAMURA[1)], and Yoshihisa UENOYAMA[1)]

[1)]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[2)]*Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki 444-8787, Japan*
[3)]*Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya 464-8601, Japan*

Abstract. Hypothalamic arcuate (ARC) kisspeptin neurons are considered the gonadotropin-releasing hormone pulse generator in rats. In virgin rats, the expression of the ARC kisspeptin gene (*Kiss1*) is repressed by proestrous levels of estradiol-17β (high E2) but not by diestrous levels of E2 (low E2). In lactating rats, ARC *Kiss1* expression is repressed by low E2 during late lactation. This study aimed to investigate whether nuclear receptor corepressor 2 (NCOR2, encoded by *Ncor2*), an estrogen receptor α corepressor, is involved in the estrogen-induced repression of ARC *Kiss1* expression in rats. Double *in situ* hybridization for *Kiss1* and *Ncor2* revealed that approximately 80% of ARC *Kiss1*-expressing cells co-expressed *Ncor2* in ovariectomized (OVX) + low E2 virgin rats, while approximately 90% of ARC *Kiss1*-expressing cells co-expressed *Ncor2* in OVX + low E2 lactating rats. To further examine the role of *Ncor2*, we studied the effects of *Kiss1*-dependent *Ncor2* knockdown on ARC *Kiss1* expression and luteinizing hormone (LH) pulses. An adeno-associated virus vector carrying Cre-activated short hairpin RNA (shRNA) for *Ncor2* was administered to the ARC in two *Kiss1-Cre* rat models: OVX + high E2 *Kiss1-Cre* virgin rats and OVX + low E2 *Kiss1-Cre* lactating rats. *Ncor2*-shRNA treatment significantly increased the number of ARC *Kiss1*-expressing cells and the intensity of *Kiss1* signals in OVX + high E2 virgin rats but failed to fully restore low E2-induced *Kiss1* repression in lactating rats. The *Ncor2*-shRNA treatment failed to affect LH pulses in both models. These findings suggest that NCOR2 in ARC kisspeptin neurons mediates high E2-induced repression of ARC *Kiss1* expression in virgin rats.

Key words: Estrogen negative feedback, Kisspeptin, Lactation, NCOR2, Reproduction

(**J. Reprod. Dev. 71:** 71–84, 2025)

Accumulating evidence suggests that hypothalamic kisspeptin neurons are master regulators of reproduction via direct stimulation of gonadotropin-releasing hormone (GnRH) and gonadotropin release in mammalian species, such as rodents [1–4], ruminants [5–7], non-human primates [8–10], and humans [11]. Specifically, kisspeptin neurons located in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV)/preoptic area (POA) are suggested to serve as GnRH pulse and surge generators, respectively, in female rodents [12–16], pigs [17], ruminants [18, 19], and primates [8, 9, 11]. Importantly, a majority of ARC and AVPV kisspeptin neurons express estrogen receptor α (ERα) in rodents [3, 12, 20, 21] and ruminants [22]. Kisspeptin gene (*Kiss1*) expression in the ARC is downregulated by circulating estrogen; thus, the ARC *Kiss1* expression level is lowest at the proestrous stage and highest at the diestrous stage in ovary-intact female rodents [3, 12, 20, 23]. Therefore, ARC kisspeptin neurons act as sites of estrogen-negative feedback to suppress GnRH/luteinizing hormone (LH) pulses. Notably, proestrous levels of estradiol-17β (high E2) strongly repress ARC *Kiss1* expression; however, diestrous levels of E2 (low E2) fail to repress the expression in ovariectomized (OVX) rats [12]. Furthermore, in lactating rats, low E2 represses ARC *Kiss1* and LH pulses during late lactation (later than 10 days after parturition during the 20 days of lactation) [24]. Contrary to ARC kisspeptin neurons, AVPV/POA kisspeptin neurons act as sites of estrogen-positive feedback to trigger GnRH/LH surges and consequent ovulation in rodents and primates because AVPV/POA *Kiss1* expression is upregulated by estrogen in rodents [12, 20, 21, 25] and Japanese monkeys [26]. ERα mediates the estrogen-induced downregulation and upregulation of *Kiss1* expression in kisspeptin neurons in the ARC and AVPV, respectively [12, 20, 21, 27], because estrogen failed to repress or enhance *Kiss1* expression in the ARC or AVPV, respectively, in OVX *Esr1* (encoding ERα) knockout mice [20]. The ERα-dependent repression or activation of ERα target genes is often mediated by ERα coregulators, such as corepressors or coactivators for the transcriptional activity of ERα [28, 29].

In the present study, we found that the nuclear receptor corepressor 2 (NCOR2) gene (*Ncor2*), an ERα corepressor gene, was highly expressed in the ARC kisspeptin neurons using RNA-seq data of isolated visualized kisspeptin neurons obtained from the ARC of female *Kiss1-tdTomato* heterozygous rats [30]. NCOR2 (also known as SMRT, which stands for silencing mediator of retinoic acid and thyroid hormone receptor) is a classic nuclear receptor corepressor that binds to ERα and serves as an adaptor to recruit proteins possessing histone deacetylase (HDAC) activity, which is known to repress gene expression by maintaining chromatin in a more condensed state [28, 29]. Notably, our previous study demonstrated that high E2 decreases histone acetylation of the *Kiss1* promoter region in

Received: November 14, 2024
Accepted: December 24, 2024
Advanced Epub: January 27, 2025

Correspondence: Y Uenoyama (e-mail: uenoyama@nagoya-u.jp)

the ARC of female mice, whose ARC *Kiss1* expression is strongly repressed, and that inhibition of histone deacetylation enhances *in vitro* *Kiss1* expression in non-*Kiss1*-expressing immortalized mouse cell lines [31]. Therefore, NCOR2 may be involved in high E2-induced repression of ARC *Kiss1* expression in rodents. In contrast, in lactating rats, low E2 is sufficient to repress ARC *Kiss1* expression and LH pulses during late lactation, as ARC *Kiss1* expression and LH pulses were suppressed in ovary-intact or low E2-treated OVX lactating rats but not in OVX lactating rats during late lactation [24]. These findings suggest that the high E2-induced repression of ARC *Kiss1* expression in cyclic rats and low E2-dependent lactation-induced repression of ARC *Kiss1* expression may differ in terms of the involvement of NCOR2.

The present study aimed to investigate whether NCOR2 is involved in the estrogen-induced repression of ARC *Kiss1* expression using two rat models: proestrous model rats that were OVX and treated with high E2, and OVX lactating rats treated with low E2. We first examined the co-expression of the *Ncor2* in ARC and AVPV kisspeptin neurons using double *in situ* hybridization (ISH) for *Ncor2* and *Kiss1* in OVX wild-type virgin and lactating rats treated with low E2. We examined the effects of *Kiss1*-dependent *Ncor2* knockdown on ARC *Kiss1* expression and pulsatile LH release by introducing an adeno-associated virus (AAV) vector carrying Cre-activated short hairpin RNA (shRNA) against *Ncor2* into the ARC of OVX *Kiss1-Cre* virgin rats treated with high E2 and OVX *Kiss1-Cre* lactating rats treated with low E2 during late lactation, during which *Kiss1* and LH pulses are largely suppressed [12, 24].

Materials and Methods

Animals

Kiss1-tdTomato heterozygous female rats [4], wild-type Wistar–Imamichi female rats (Institute for Animal Reproduction, Kasumigaura, Japan), and *Kiss1-Cre* female rats [32] were used in this study. *Kiss1-tdTomato* heterozygous and *Kiss1-Cre* rats were raised in our laboratory and validated in our previous studies [4, 32]. Animals were maintained in a room with a 14/10 h light/dark cycle (lights on 0500 h) at 22 ± 3°C and had free access to food (CE-2; CLEA Japan, Tokyo, Japan) and water. Animals (8–13 weeks of age) with two consecutive regular 4-day estrous cycles, as determined by daily observation of vaginal smears, were used. *Kiss1-tdTomato* heterozygous female rats, whose coding sequences for *Kiss1* exons 2 and 3 were replaced with the *tdTomato* gene, were subjected to RNA-seq analysis, as described previously [30]. Wild-type Wistar–Imamichi female rats were subjected to RT-PCR analysis to examine ARC *Ncor2* expression and histological analysis to determine *Ncor2* expression in *Kiss1*-expressing cells in the ARC and AVPV. *Kiss1-Cre* female rats, whose coding sequences for *Kiss1* exons 2 and 3 were replaced with *Cre* recombinase [32], were used for *Kiss1*-dependent *Ncor2* knockdown in the ARC.

Kiss1-tdTomato and wild-type female rats were bilaterally OVX, and some were treated with low or high E2. Briefly, the animals were OVX for 2 weeks to serve as the OVX group. Some OVX rats were immediately treated with a subcutaneous Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 25 mm in length; Dow Corning, Midland, MI, USA) filled with E2 (Sigma-Aldrich, St. Louis, MO, USA), dissolved in peanut oil (Sigma-Aldrich) at 20 µg/ml, for 1 week to serve as OVX + low E2 rats, in which plasma E2 levels mimicked the diestrous level [33]. Some OVX rats were treated with a subcutaneous Silastic tubing filled with low E2 for 5 days, followed by a Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 28 mm in length) filled with high E2, dissolved in peanut oil at 1,000 µg/ml, for 2 days to serve as the OVX + high E2 rats, in which plasma E2 levels mimicked the proestrous level [34].

Wild-type or *Kiss1-Cre* lactating rats were obtained by mating with a male overnight on the day of proestrus, and pregnant females were housed individually. The day of parturition was designated day 0 postpartum, and the litter size of the lactating rats was adjusted to eight (four males and four females) on day 1 postpartum. Non-lactating rats were obtained by depriving all pups on day 1 postpartum. On day 2 postpartum, all animals were bilaterally OVX, and some OVX lactating rats were immediately implanted with a Silastic tubing containing low E2. The E2-containing tubing was replaced with new tubing on day 9 postpartum to maintain a constant plasma E2 level.

The surgical operations were performed under anesthesia with an intraperitoneal injection of ketamine (27 mg/kg; Fujita, Tokyo, Japan) and xylazine (5.3 mg/kg; Bayer AG, Leverkusen, Germany) mixture, followed by inhalation of isoflurane (1–3%, Pfizer Japan, Tokyo, Japan). The care of animals and all experimental procedures performed in the present study were reviewed and approved by the Animal Experiment Committee of Nagoya University.

RNA-seq analysis of ERα corepressor candidates in isolated tdTomato-positive cells obtained from Kiss1-tdTomato heterozygous female rats

The expression profiles of ERα corepressor genes in ARC or AVPV kisspeptin neurons were analyzed using RNA-seq data obtained from *Kiss1-tdTomato* heterozygous rats, as described in our previous study [30]. Briefly, ARC and AVPV tissues were dissected from OVX and OVX + high E2 *Kiss1-tdTomato* heterozygous rats, respectively, because tdTomato signals were visible in the ARC under the OVX condition and in the AVPV under the OVX + high E2 condition. The tdTomato-positive cells were isolated and picked under a fluorescence microscope using a glass pipette. Ten (for ARC) and three (for AVPV) tdTomato-positive cells were pooled and subjected to RNA-seq analysis. mRNA expression levels were normalized by calculating the reads per kilobase per million mapped reads for each mRNA [30].

Quantitative RT-PCR analysis for Ncor2 mRNA expression levels in the ARC of virgin female rats

To examine the effects of E2 on ARC *Ncor2* mRNA expression, wild-type OVX, OVX + low E2, and OVX + high E2 rats (n = 5 in each group) were decapitated between 1300 and 1500 h, as described previously [34], and *Ncor2* mRNA levels in the ARC of each group were quantified by RT-PCR. Briefly, the ARC-median eminence (ME) region was dissected from the brain using a microblade, and DNA-free total RNA was purified from the ARC-ME tissue using ISOGEN (Nippon Gene, Tokyo, Japan). The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize full-length cDNAs. The obtained cDNA was used as a template in PCR reactions using primers for *Ncor2* (5'-ctctcgctgtggaccctaag-3' and 5'-cctgctgatggtaccttgt-3') and *Actb* (5'-tgtcaccaactgggacgata-3' and 5'-ggggtgttgaaggtctcaaa-3'). Quantitative RT-PCR analysis of *Ncor2* mRNA expression was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) with THUNDERBIRD qPCR Mix (TOYOBO, Osaka, Japan), as described previously [34]. The expression levels of *Ncor2* mRNA were normalized to those of *Actb* mRNA.

Histological analysis of co-expression of Ncor2 in the hypothalamic kisspeptin neurons of OVX + low E2 virgin and lactating rats

Free-floating double ISH for *Kiss1* and *Ncor2* was performed using the hypothalamic sections, including the ARC (from 1.72 to 4.36 mm posterior to the bregma, every fourth section, 13 sections in total) or the AVPV (from 0.12 mm anterior to 0.60 mm posterior to the bregma, every second section, eight sections in total) of wild-type virgin OVX + low E2 rats, as previously described [30, 35, 36]. OVX + low E2 virgin rats were selected because OVX + low E2 rats have been confirmed to show *Kiss1* expression in both the ARC and AVPV [12, 30, 34]. Briefly, the animals were perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (Sigma-Aldrich) in 0.05 M phosphate buffer, and the brains were dissociated and post-fixed with the same fixative overnight. The fixative was replaced with 30% sucrose in 0.05 M phosphate buffer, and brain sections were made by a cryostat (CM1800, Leica Biosystems, Wetzlar, Germany). The sections were hybridized overnight at 60°C with 1 μg/ml fluorescein isothiocyanate (FITC)-labeled *Kiss1* anti-sense cRNA probe (position 33-348, AY196983) [37] and digoxigenin (DIG)-labeled *Ncor2* anti-sense cRNA probe (position 7196-8366, NM_001108334.1). The FITC-labeled *Kiss1* probe on the sections was visualized by peroxidase (POD)-conjugated anti-FITC antibody (RRID: AB_840257, 1:1000, Roche Diagnostics, Basel, Switzerland) and tyramide signal amplification (TSA) Plus FITC Kit (1:100; PerkinElmer, Shelton, CT, USA). After the POD inactivation on the POD-conjugated anti-FITC antibody with 0.1 N hydrochloric acid for 30 min, the DIG-labeled *Ncor2* probe on the sections was visualized by a POD-conjugated anti-DIG antibody (RRID: AB_514500, 1:1000, Roche Diagnostics), TSA Plus Biotin Kit (1:100; PerkinElmer), and DyLight 594-conjugated streptavidin (1:500, Thermo Fisher Scientific). No positive signals for *Ncor2* mRNA were detected in brain sections hybridized with the corresponding sense probe as a negative control (data not shown). The sections were mounted on slides, and fluorescent images were obtained under a fluorescence microscope with ApoTome2 optical sectioning (Carl Zeiss, Oberkochen, Germany). *Kiss1*- and *Ncor2*-positive cells were counted unilaterally on the photomicrographs, according to the rat brain atlas [38].

Free-floating double ISH for *Kiss1/Ncor2* or *Tac3* (neurokinin B gene)/*Ncor2* was performed as described above using hypothalamic sections including the ARC of OVX + low E2 lactating rats. Brains were collected on day 16 postpartum according to our previous study, which showed that ARC *Kiss1* expression was repressed in lactating rats in a low E2-dependent manner during late lactation [24]. *Tac3* mRNA was used as an indicator of ARC kisspeptin neurons in lactating rats because the majority of ARC kisspeptin neurons co-express neurokinin B in rodents and ruminants [39–41], and *Tac3* mRNA was comparably expressed in lactating and non-lactating rats on day 8 postpartum [42]. The sections were hybridized with FITC-labeled *Kiss1* or *Tac3* anti-sense cRNA probe (position 180–483, NM_019162) [43] and DIG-labeled *Ncor2* anti-sense cRNA probe. The hybridized probes were detected and analyzed as described above.

In vitro validation of Ncor2-siRNA candidates to repress Ncor2 mRNA expression and NCOR2 protein biosynthesis using mHypoA-55 cell line, a model for rodent ARC kisspeptin neurons

Two candidate sequences of *Ncor2*-siRNA (anti-sense sequences, including the overhang; siRNA #1, AGACUUUGGUUCCAAAUGCgg; siRNA #2, UUAACUCAUUGAAGGUAUCaa) were designed using siDirect version 2.0 (http://sidirect2.rnai.jp/) with the rat *Ncor2* mRNA sequence. These candidate sequences were compatible with rat and mouse *Ncor2* mRNA and were matched to generate shRNA. Additionally, we selected two *Ncor2*-siRNA (siRNA #3, UUAGUUAAGGCUUUAGACAgg; siRNA #4, AUUUACCCAUGAGUGCCUUuc) as previously reported [44]. The four *Ncor2*-siRNA candidates were synthesized and duplexed by Sigma-Aldrich. The MISSION siRNA Universal Negative Control #1 (siRNA-NC, Sigma-Aldrich) was used as a negative control for *in vitro* validation of *Ncor2*-siRNA candidates.

Immortalized mouse neuronal cells, mHypoA-55 cells (RRID: CVCL_D416; CELLutions Biosystems, Burlington, ON, Canada), were used as a model for rodent ARC kisspeptin neurons [34] to validate the silencing effect of *Ncor2*-siRNA candidates *in vitro*, as described in our previous study [34]. One of four *Ncor2*-siRNA candidates or siRNA-NC (10 nM) was transfected into mHypoA-55 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). After transfection, the cells were cultured for another 24 or 48 h. The cells were treated with E2 (100 nM) for 2 h and then harvested for the analysis of *Ncor2* mRNA levels (n = 4). Total RNA was purified from harvested cells using ISOGEN. ReverTra Ace (TOYOBO, Osaka, Japan) was used to synthesize full-length cDNAs, as described previously [34]. The obtained cDNA was used as a template in PCR reactions using primers for *Ncor2* (5'-ctgggacggaaatcttcaac-3' and 5'-ggcattcagagggttaaaagc-3') and *Actb* (5'-ggtgggaatgggtcagaagg-3' and 5'-gtacatggctggggtgttga-3'). The relative expression levels of *Ncor2* mRNA were normalized to those of *Actb* mRNA.

The siRNA #1, siRNA #2, or siRNA-NC were transfected into the mHypoA-55 cells in a similar manner. Whole-cell lysates were collected 48 h after transfection for analysis of NCOR2 protein levels (n = 4). The lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA), as previously described [34]. The membranes were incubated with rabbit anti-human SMRT polyclonal antibody (RRID: AB_310286; 1:2000, Millipore, Burlington, MA, USA) at 4°C for 16 h. The specificity of the antibody has been confirmed previously [45]. The membranes were then incubated with horseradish POD-conjugated goat anti-rabbit IgG antibody (RRID: AB_955447; 1:10000, Abcam, Cambridge, UK) at room temperature for 1 h. Chemiluminescence was visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and recorded with Light-Capture II (AE-6982; ATTO, Tokyo, Japan). The membranes were treated with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) and incubated with rabbit anti-human β-actin polyclonal antibody (RRID: AB_2305186, 1:10000; Abcam, Cambridge, UK). The expression of the β-actin protein was detected using the method described above. Band intensities were estimated by densitometry using ImageJ version 1.53k (https://imagej.nih.gov/ij/). The relative expression levels of NCOR2 protein were normalized by the levels of β-actin protein.

Preparation of AAV carrying Ncor2 shRNA

AAV-DJ vectors carrying shRNA #1 against *Ncor2* mRNA were prepared, as previously described [46]. DNA constructs encoding Cre-dependent *Ncor2*-targeted shRNA #1 or its scrambled-shRNA were designed (Table 1) and synthesized in the pMK plasmid using the GeneArt Strings DNA Fragments service (Thermo Fisher Scientific). The pMK plasmid backbone was replaced with the AAV-U6-EGFP plasmid (Vector Biolabs, Malvern, PA, USA). The AAV-U6-EGFP vector contains an EGFP-expressing sequence under the control of a cytomegalovirus (CMV) promoter. The loop sequence, including the floxed stop cassette in the DNA constructs, was obtained from a

Table 1. DNA sequences of rat *Ncor2*-targeted shRNA #1 or its scrambled shRNA

Ncor2-targeted shRNA #1	gcatttggaaccaaagtctgaagctataacttcgtatagcatacattatacgaagttatagcttggtagcgcg gtgtattatacttttttggaaagaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgtta cccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccg atcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcctgatgcggtattttctccttacgcatc tgtgcggtatttcacaccgcatatttttttataacttcgtatagcatacattatacgaagttatagcttgAGAC TTTGGTTCCAAATGCttttttt
scrambled shRNA	aggccgtattaacgtactagaagctataacttcgtatagcatacattatacgaagttatagcttggtagcgcg gtgtattatacttttttggaaagaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgtta cccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccg atcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcctgatgcggtattttctccttacgcatc tgtgcggtatttcacaccgcatatttttttataacttcgtatagcatacattatacgaagttatagcttgTAGT ACGTTAATACGGCCTttttttt

The DNA sequences for the sense and anti-sense strands of shRNA targeting rat *Ncor2* and its scrambled shRNA are shown, with the sense strand in lowercase letters and the anti-sense strand in uppercase letters, both highlighted in blue. The DNA sequences for the two loxP sites are indicated with underlines.

previous study [47]. The resulting AAV-U6-shRNA expressed shRNA under the control of the U6 promoter, an RNA polymerase III (Pol III) promoter, in a Cre-dependent manner. The U6 promoter was selected because the U6 promoter has been widely used for shRNA expression [47–49]. In addition, the previous study [47] suggested that the U6 promoter is suitable for Cre-mediated shRNA expression compared with other Pol III promoters, such as the H1 promoter. Human embryonic kidney (HEK) 293T cells were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C and 5% CO_2 in air. The AAV-DJ capsid plasmid (Cell Biolabs, San Diego, CA, USA), pHelper (Cell Biolabs), and AAV-U6-shRNA were co-transfected into HEK293T cells using polyethyleneimine transfection reagent (Sigma-Aldrich). Three days after the transfection at 37°C and 3% CO_2 in air, virus vectors were purified by a gradient purification method using iodixanol OptiPrep (Serumwerk Bernburg, Bernburg, Germany), and the titer was quantified using quantitative PCR.

Effects of ARC Kiss1-dependent Ncor2 knockdown on ARC Kiss1 expression and LH secretion in OVX + high E2 Kiss1-Cre virgin rats and OVX + low E2 Kiss1-Cre lactating rats

AAV-DJ vectors were stereotaxically injected into the ARC of *Kiss1-Cre* female rats, as described previously [43]. Briefly, AAV vectors carrying U6 promoter-driven Cre-activated *Ncor2*-targeting shRNA #1 (AAV-U6-[*Ncor2*-shRNA #1 sense-loxP-stop-loxP-*Ncor2*-shRNA #1 anti-sense]-CMV-EGFP, 1.16×10^8 viral genomes [vg]/µl) or scrambled-shRNA control (AAV-U6-[Scrambled-shRNA sense-loxP-stop-loxP-Scrambled-shRNA anti-sense]-CMV-EGFP, 0.32×10^8 vg/µl) were bilaterally injected into the anterior and posterior ARC of *Kiss1-Cre* virgin cyclic female rats (*Ncor2*-shRNA #1, n = 8; scrambled-shRNA, n = 6) or *Kiss1-Cre* lactating rats (*Ncor2*-shRNA #1, n = 8; scrambled-shRNA, n = 4) on day 2 of lactation under anesthesia with a mixture of medetomidine (0.375 mg/kg, ZENOAQ, Koriyama, Japan), midazolam (2 mg/kg, SANDOZ, Tokyo, Japan), and butorphanol (2.5 mg/kg, Meiji Animal Health, Kumamoto, Japan). The stereotaxic coordinates for the injections were 2.8 mm (anterior ARC) and 3.8 mm (posterior ARC) posterior, 10.3 mm ventral to the bregma, and 0.6 mm lateral to the midline, based on the rat brain atlas [38]. Each of the four bilateral injection sites received 1 µl of the vector at a flow rate of 0.25 µl/min over 4 min. After the surgery, the animals were awakened with an injection of atipamezole (0.75 mg/kg, ZENOAQ), a medetomidine antagonist. Three weeks after the AAV injection, *Kiss1-Cre* virgin female rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA were subjected to high E2 treatment, as described earlier. OVX + high E2 *Kiss1-Cre* virgin rats were subjected to blood sampling to determine LH pulses. OVX + low E2 *Kiss1-Cre* lactating rats treated with AAV on day 2 postpartum were subjected to blood sampling on day 16 postpartum to assess LH pulses. On the day before blood sampling, a silicon cannula (inner diameter 0.5 mm, outer diameter 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) was inserted into the right atrium via the jugular vein in the AAV-treated *Kiss1-Cre* virgin and lactating rats. Blood samples (100 µl) were collected from freely moving conscious OVX + high E2 *Kiss1-Cre* virgin female rats every 6 min for 3 h from 1000 to 1300 h to avoid detecting the afternoon LH surge. Blood samples were similarly collected from OVX + low E2 *Kiss1-Cre* lactating rats from 1300 to 1600 h at 6-min intervals, as previously described [24]. An equivalent volume of rat red blood cells, obtained from donor rats and diluted with heparinized saline, was replaced through the atrial catheter after each blood collection to maintain a constant hematocrit. Plasma samples were isolated following centrifugation at 4°C.

After blood sampling, the animals were perfused with PBS followed by 4% paraformaldehyde. The brains were collected and sectioned. The sections including the ARC region (from 1.72 to 4.36 mm posterior to the bregma, every fourth section, 13 sections in total) were subjected to ISH for ARC *Kiss1* mRNA expression, as previously described [12]. The sections were hybridized with a 1 µg/ml DIG-labeled *Kiss1* anti-sense cRNA probe at 60°C overnight. The hybridized probe was detected with an alkaline phosphatase-conjugated anti-DIG antibody (RRID: AB_2734716; 1:1000; Roche Diagnostics) for 3 h at 37°C. The sections were treated with a chromogen solution (338 µg/ml 4-nitroblue tetrazolium chloride and 175 µg/ml 5-bromo-4-chloro-3-indoyl-phosphate) for 3 h at 37°C.

The brain sections were mounted, and the signals were examined using an optical microscope (BX53; Olympus, Tokyo, Japan). The number of *Kiss1*-expressing cells in the ARC was counted bilaterally in duplicate and averaged. The intensity of *Kiss1* mRNA signals in digital photographs of the ARC was quantitatively analyzed using Fiji software (ImageJ, version 2.1.0/1.53c), as previously described [50, 51].

Double staining for Tac3 and EGFP using ISH for Tac3 and immunohistochemistry for EGFP

To evaluate the transfection efficacy of AAV vectors in the ARC kisspeptin neurons, ISH for *Tac3* mRNA and immunohistochemistry

for EGFP were performed using brain sections from OVX + high E2 *Kiss1-Cre* virgin rats and OVX + low E2 *Kiss1-Cre* lactating rats treated with AAV, as previously described [52]. Another series of brain sections, including the ARC region (from 1.72 to 4.36 mm posterior to the bregma, every fourth section, 13 sections in total), were incubated with an anti-EGFP antibody (RRID: AB_300798, 1:1000; Abcam) for two nights at 4°C. The brain sections were then fixed with 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chemical, Osaka, Japan) for 10 min and rinsed with 0.05 M PBS containing 0.2% glycine. The sections were incubated with 1 µg/ml DIG-labeled *Tac3* cRNA probe overnight at 60°C, as described previously [43]. The DIG-labeled *Tac3* signal was visualized using the POD-conjugated anti-DIG antibody, TSA Plus Biotin Kit (1:100, Akoya Bioscience, Marlborough, MA, USA), and DyLight 594-conjugated streptavidin (1:500, Thermo Fisher Scientific). The anti-EGFP signal was visualized using Alexa Fluor 488 goat anti-chicken IgY secondary antibody (RRID: AB_2534096, 1:800; Thermo Fisher Scientific) for 2 h at room temperature. Fluorescent images were obtained under a fluorescence microscope with ApoTome2 optical sectioning (Carl Zeiss, Oberkochen, Germany). *Tac3*- and EGFP-positive cells were counted bilaterally on the photomicrographs.

Radioimmunoassay and LH pulse parameter analysis

Plasma LH concentrations were determined using a double-antibody radioimmunoassay (RIA) with a rat LH-RIA kit provided by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA). Concentrations were expressed in terms of rat LH-RP-3 (National Institute of Diabetes and Digestive and Kidney Diseases). The least detectable level in the LH assay was 3.9 pg/tube, and the intra- and inter-assay coefficients of variation were 6.18% and 11.26% at 0.625 ng/ml, respectively. LH pulses were identified using the PULSAR computer program [53]. The mean LH concentration and the baseline, frequency, and amplitude of LH pulses were calculated during the 3 h sampling period for each individual and then for the group.

Statistical analysis

Statistical differences in ARC *Ncor2* mRNA levels among OVX, OVX + low E2, and OVX + high E2 rats were determined by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. Statistical differences in the number of *Kiss1*- or *Tac3*-expressing cells and *Kiss1/Ncor2*- or *Tac3/Ncor2*-co-expressing cells among OVX, OVX + low E2 lactating, and OVX + low E2 non-lactating rats, as well as *Ncor2* mRNA and NCOR2 protein levels between the mHypoA-55 cells treated with *Ncor2*-siRNA candidates and siRNA-NC, were determined by one-way ANOVA followed by Tukey's HSD test. Statistical differences in the numbers of *Tac3*-expressing cells and *Tac3*/EGFP-double-positive cells, the percentage of *Tac3*-positive cells co-expressing EGFP, the number of *Kiss1*-expressing cells, the intensity of *Kiss1* mRNA signals, and the LH pulse parameters (the mean LH concentration, as well as the baseline, frequency, and amplitude of LH pulses) between *Ncor2*-shRNA #1- and scrambled-shRNA-treated OVX + high E2 *Kiss1-Cre* rats and OVX + low E2 *Kiss1-Cre* lactating rats were determined using Student's *t*-test.

Results

Ncor2 expression in ARC and AVPV kisspeptin neurons in Kiss1-tdTomato heterozygous female rats

RNA-seq analysis of kisspeptin neurons isolated from OVX *Kiss1-tdTomato* heterozygous rats revealed that *Ncor2* and *Ncor1* were highly expressed in ARC kisspeptin neurons, whereas other corepressor genes, such as *Phb2*, *Nrip1*, and *Siah2*, were scarcely detected (Figs. 1A, B). The mRNA expression of these genes, including *Ncor2* and *Ncor1*, was barely detectable in the AVPV kisspeptin neurons of OVX + high E2 *Kiss1-tdTomato* heterozygous rats (Fig. 1B). Quantitative RT-PCR analysis using wild-type female rats showed that ARC *Ncor2* mRNA expression levels were comparable among the OVX, OVX + low E2, and OVX + high E2 groups (Figs. 1C, D; $P > 0.05$).

Double ISH for *Ncor2* and *Kiss1* showed that *Ncor2* mRNA was expressed in a majority of ARC *Kiss1*-expressing cells of OVX + low E2 rats (Fig. 1E). Quantitative analysis revealed that 82.6 ± 1.0% of ARC *Kiss1*-expressing cells exhibited *Ncor2* mRNA in OVX + low E2 wild-type rats (Fig. 1F). *Ncor2* mRNA expression was also found in the AVPV *Kiss1*-expressing cells of OVX + low E2 rats (Fig. 1G). Quantitative analysis revealed that 43.0 ± 1.3% of AVPV *Kiss1*-expressing cells exhibited *Ncor2* mRNA in OVX + low E2 wild-type rats (Fig. 1H). Since the proportion of kisspeptin neurons expressing *Ncor2* was much higher in ARC kisspeptin neurons than in AVPV kisspeptin neurons, the role of NCOR2 in the estrogen-dependent regulation of ARC *Kiss1* expression and GnRH/LH pulse generation in female rats was further investigated.

Ncor2 expression in ARC kisspeptin neurons during late lactation determined by double ISH

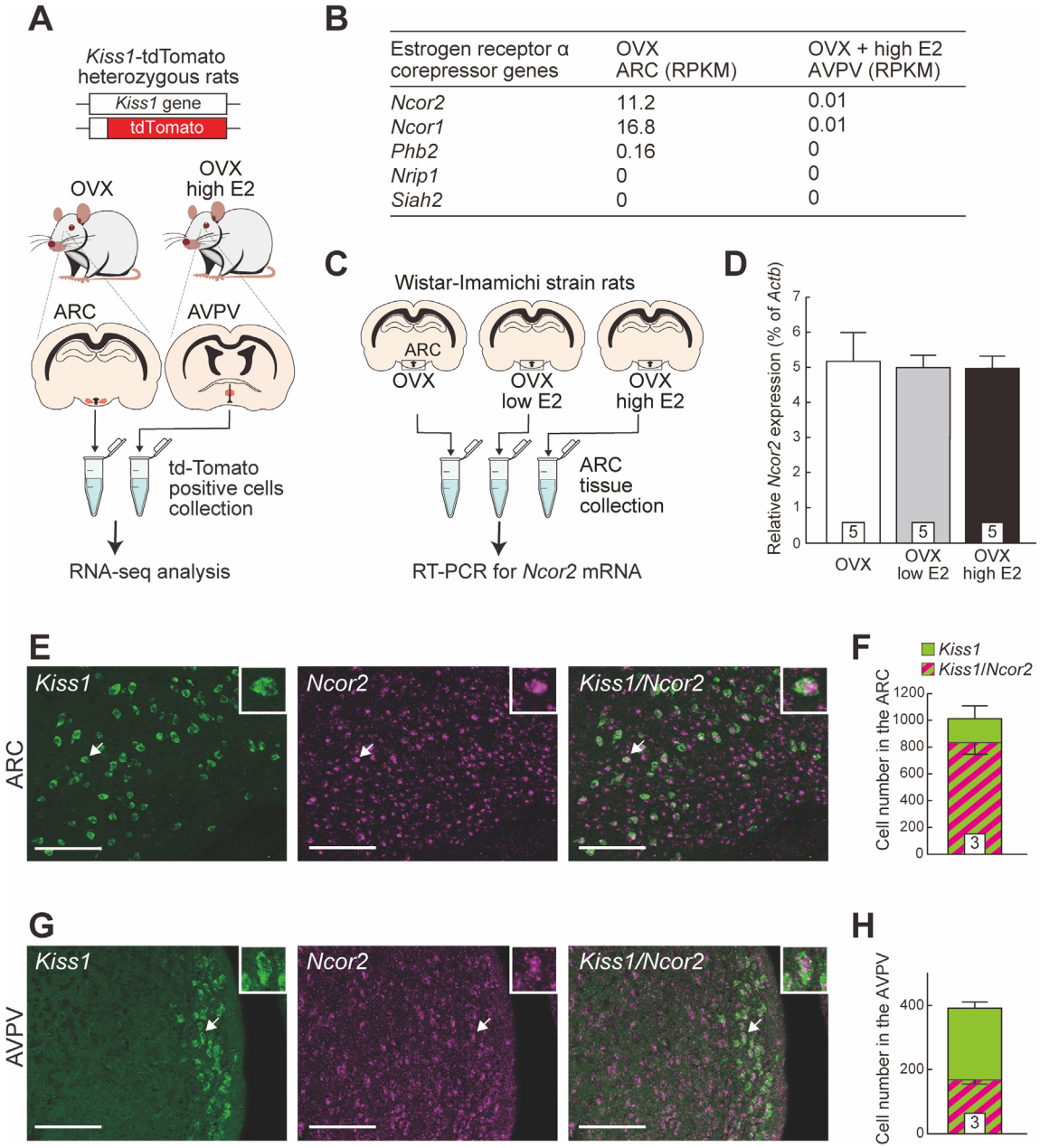
The expression of *Ncor2* in ARC kisspeptin neurons was analyzed in OVX + low E2 non-lactating rats, OVX + low E2 lactating rats, and OVX lactating rats during late lactation (Fig. 2A). Many ARC *Kiss1*-expressing cells were found in OVX lactating and OVX + low E2 non-lactating rats, whereas OVX + low E2 lactating rats showed fewer ARC *Kiss1*-expressing cells compared to the other groups (Fig. 2B). *Ncor2* mRNA was observed in the majority of *Kiss1*-expressing cells in all three groups, and the percentages of *Kiss1/Ncor2*-co-expressing cells among the total ARC *Kiss1*-expressing cells were 89.0 ± 2.5% in OVX + low E2 non-lactating rats, 80.2 ± 1.9% in OVX + low E2 lactating rats, and 92.1 ± 1.6% in OVX lactating rats (Fig. 2C).

Quantitative analysis revealed that the number of ARC *Kiss1*-expressing cells was significantly lower in OVX + low E2 lactating rats compared to OVX + low E2 non-lactating ($P < 0.0001$) and OVX lactating rats ($P < 0.0001$). Additionally, the number of ARC *Kiss1*-expressing cells was significantly lower in OVX lactating rats compared to OVX + low E2 non-lactating rats ($P = 0.0272$). The number of ARC *Kiss1/Ncor2*-co-expressing cells was also significantly lower in OVX + low E2 lactating rats compared to OVX + low E2 non-lactating ($P < 0.0001$) and OVX lactating rats ($P < 0.0001$).

Many ARC *Tac3* (an indicator of ARC kisspeptin neurons)-expressing cells were observed in all three groups (Fig. 2D). The numbers of ARC *Tac3*-expressing cells and *Tac3/Ncor2*-co-expressing cells were comparable between OVX + low E2 non-lactating, OVX + low E2 lactating, and OVX lactating rats (Fig. 2E; $P > 0.05$). The percentages of *Tac3/Ncor2*-co-expressing cells among the total ARC *Tac3*-expressing cells were 87.8 ± 0.6% in OVX + low E2 non-lactating rats, 90.3 ± 1.0% in OVX + low E2 lactating rats, and 88.4 ± 2.0% in OVX lactating rats (Fig. 2E).

Validation of Ncor2 mRNA and protein repression by Ncor2 silencing in mHypoA-55 cell line

Four *Ncor2*-targeting siRNA candidates (siRNAs #1–#4) were used for *in vitro* evaluation of their silencing effects (Fig. 3A). Quantitative



Estrogen receptor α corepressor genes	OVX ARC (RPKM)	OVX + high E2 AVPV (RPKM)
Ncor2	11.2	0.01
Ncor1	16.8	0.01
Phb2	0.16	0
Nrip1	0	0
Siah2	0	0

Fig. 1. Evaluation of estrogen receptor α (ERα) corepressor candidate gene expression in kisspeptin neurons in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) and nuclear receptor corepressor 2 gene (*Ncor2*) expression in ARC and AVPV kisspeptin neurons in female rats. (A) Schematic of the procedure for RNA-seq analysis for ERα corepressors using *Kiss1-tdTomato* heterozygous female rats. The tdTomato-positive kisspeptin neurons were collected from the ARC in ovariectomized (OVX) rats and from the AVPV in OVX rats treated with proestrous levels of estradiol-17β (OVX + high E2). (B) Gene expression profiles of ERα corepressors in the tdTomato-positive ARC or AVPV *Kiss1-tdTomato* cells obtained from OVX or OVX + high E2 *Kiss1-tdTomato* heterozygous female rats. The values were normalized by the reads per kilobase per million mapped reads (RPKM) for each mRNA. (C) Schematic of the procedure for quantitative RT-PCR analysis of ARC *Ncor2* mRNA expression in the ARC using OVX, OVX + diestrous levels of E2 (OVX + low E2), and OVX + high E2 wild-type female rats. (D) Relative ARC *Ncor2* mRNA expression (normalized to the expression level of *Actb* mRNA) in OVX, OVX + low E2, and OVX + high E2 female rats. (E) Expression of kisspeptin gene (*Kiss1*, green) and *Ncor2* (magenta) mRNA in the ARC of a representative OVX + low E2 wild-type female rat, as detected by *in situ* hybridization (ISH). (F) The number of ARC cells expressing *Kiss1* alone (green column) or both *Kiss1* and *Ncor2* (green and magenta striped column). (G) Expression of *Kiss1* (green) and *Ncor2* (magenta) mRNA in the AVPV of a representative OVX + low E2 wild-type female rat. (H) The number of AVPV cells expressing *Kiss1* alone (green column) or both *Kiss1* and *Ncor2* (green and magenta striped column). The insets indicate representative *Kiss1*, *Ncor2*, and *Kiss1/Ncor2*-co-expressing cells (arrows) at higher magnification. Scale bars, 100 μm. Values expressed are mean ± SEM. The number in each column indicates the number of animals used.

RT-PCR analysis revealed that *Ncor2*-targeting siRNAs #1 and #2 significantly reduced *Ncor2* mRNA levels in the mHypoA-55 cell line at 24 h (P = 0.0128 and P = 0.0188, respectively) and 48 h (P = 0.0211 and P = 0.0228, respectively) after transfection compared to the untreated group (Fig. 3B). *Ncor2*-targeting siRNAs #3 and #4 significantly reduced *Ncor2* mRNA levels at 24 h (P = 0.0122 and P = 0.0228, respectively); however, the mRNA levels partly recovered 48 h after transfection (Fig. 3B). In addition, western blot analysis

demonstrated that *Ncor2*-targeted siRNA #1, but not siRNA #2, significantly reduced NCOR2 protein levels in mHypoA-55 cells 48 h after transfection (Fig. 3C; P = 0.0377).

Ncor2 knockdown partly blocked estrogen-induced ARC Kiss1 repression in female rats

We first investigated the effects of ARC *Kiss1*-dependent *Ncor2* knockdown using an AAV vector carrying *Ncor2*-shRNA #1,

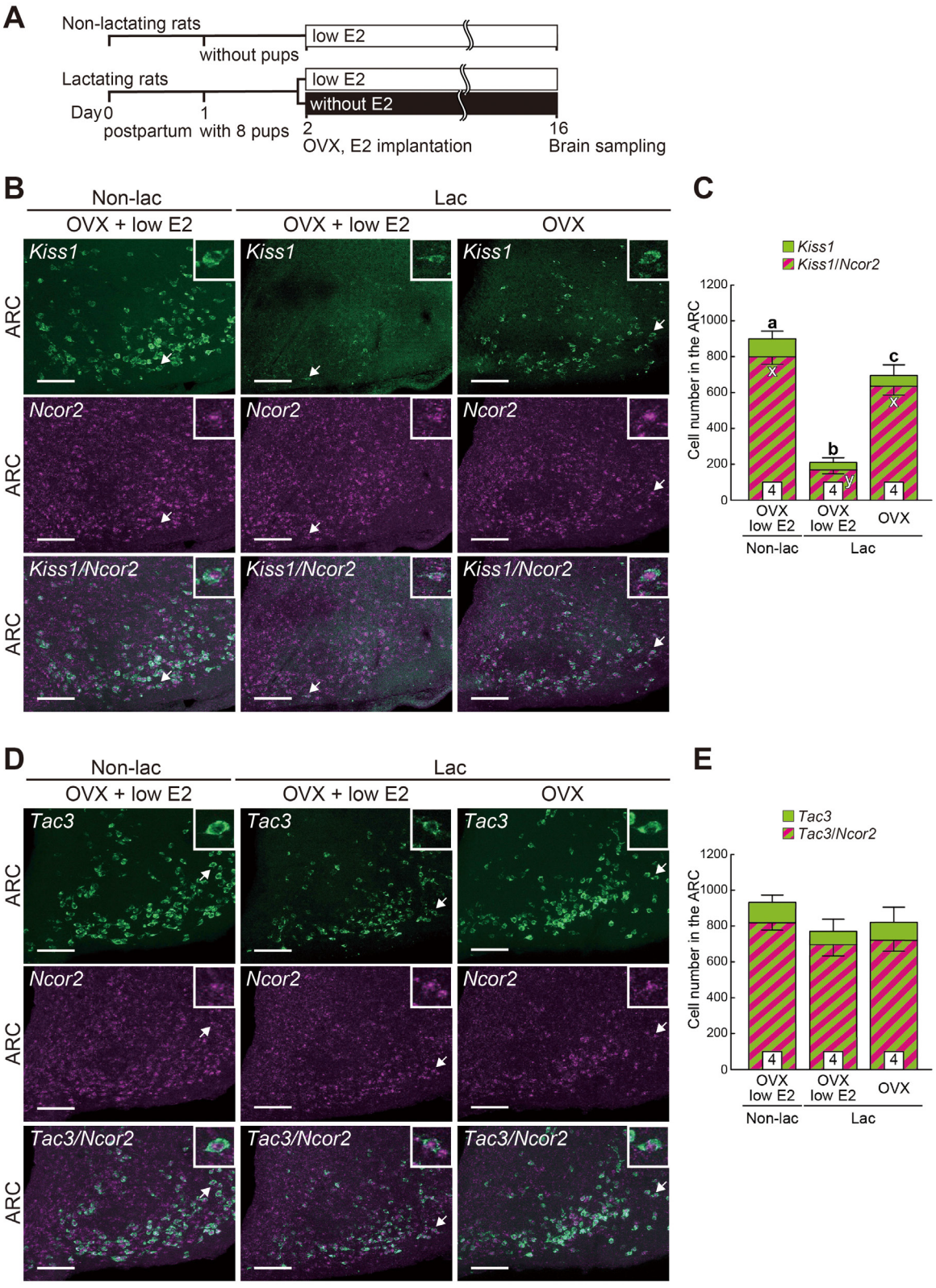


Fig. 2.

Fig. 2. Double ISH for *Kiss1* and *Ncor2* or neurokinin B gene (*Tac3*) and *Ncor2* mRNA expression in the ARC in OVX + low E2 non-lactating, OVX + low E2 lactating, and OVX lactating wild-type rats. (A) Schematic of the experimental schedule to investigate ARC *Kiss1/Tac3* and *Ncor2* mRNA expression in non-lactating and lactating rats. The day of delivery was set as day 0 postpartum. On day 1 postpartum, the number of pups was adjusted to eight for lactating rats, and all pups were deprived for non-lactating rats. On day 2 postpartum, non-lactating and lactating rats were OVX and treated with low E2 implantation, while some OVX lactating rats were kept without E2 treatment. Brain samples were collected on day 16 postpartum. (B) Expression of *Kiss1* (green) and *Ncor2* (magenta) mRNA in the ARC of representative OVX + low E2 non-lactating, OVX + low E2 lactating, and OVX lactating rats. The insets indicate representative *Kiss1*, *Ncor2*, and *Kiss1/Ncor2*-co-expressing cells (arrows) at higher magnification. (C) The number of *Kiss1*-expressing cells (green column) and the number of *Kiss1/Ncor2*-co-expressing cells (green and magenta striped column) in OVX + low E2 lactating rats were significantly lower than those in OVX + low E2 non-lactating and OVX lactating rats. Additionally, the number of *Kiss1*-expressing cells in OVX lactating rats was significantly lower than those in OVX + low E2 non-lactating rats. Values with different letters were significantly different from each other ($P < 0.05$, one-way ANOVA followed by Tukey's HSD test). (D) Expression of *Tac3* (green) and *Ncor2* (magenta) mRNA in the ARC of representative OVX + low E2 non-lactating, OVX + low E2 lactating, and OVX lactating rats. The insets indicate representative *Tac3*, *Ncor2*, and *Tac3/Ncor2*-co-expressing cells (arrows) at higher magnification. (E) The number of ARC cells expressing *Tac3* alone (green column) or both *Tac3* and *Ncor2* (green and magenta striped column) in OVX + low E2 non-lactating, OVX + low E2 lactating, and OVX lactating rats were comparable between groups. Scale bars, 100 μm. Values expressed are mean ± SEM. The number in each column indicates the number of animals used.

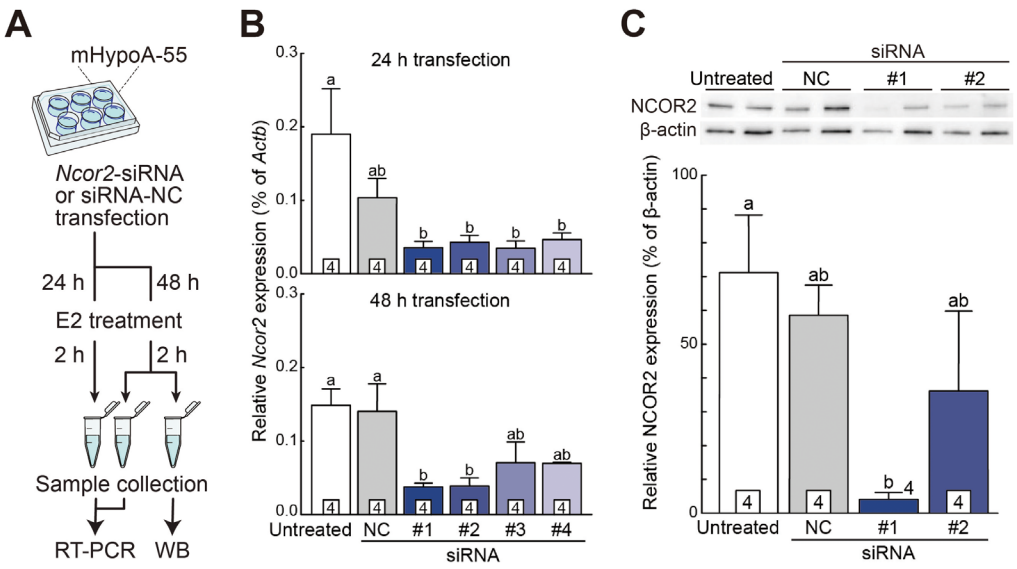


Fig. 3. *In vitro* validation of *Ncor2*-siRNA candidates to repress *Ncor2* mRNA expression and NCOR2 protein expression in the mHypoA-55 cell line. (A) Schematic of the procedure for evaluating *Ncor2*-siRNA in the mHypoA-55 cell line. mHypoA-55 cells were transfected with one of four *Ncor2* siRNA candidates (siRNA #1–#4) or The MISSION siRNA Universal Negative Control #1 (siRNA-NC). Following transfection, the cells were cultured for an additional 24 or 48 h and treated with E2 for 2 h prior to collection. (B) *Ncor2* mRNA expression was analyzed by quantitative RT-PCR 24 or 48 h after *Ncor2*-siRNA treatment, with expression levels normalized to *Actb* mRNA levels. Values with different letters were significantly different from each other ($P < 0.05$, one-way ANOVA followed by Tukey's HSD test). (C) NCOR2 and β-actin protein expression levels were analyzed by western blotting. NCOR2 protein levels were normalized to β-actin protein levels. Values with different letters were significantly different from each other ($P < 0.05$, one-way ANOVA followed by Tukey's HSD test). *Ncor2*-targeting siRNA #1 significantly reduced both *Ncor2* mRNA and NCOR2 protein levels in the mHypoA-55 cell line compared to the untreated group. The number in each column indicates the number of cell culture replicates used.

corresponding to siRNA #1, on ARC *Kiss1* expression in OVX + high E2 *Kiss1-Cre* rats (Fig. 4A). Representative images of *Tac3*/EGFP-double-positive cells in the anterior and posterior ARC of OVX + high E2 *Kiss1-Cre* virgin rats transfected with *Ncor2*-targeting shRNA #1 or scrambled-shRNA are shown in Fig. 4B. Quantitative analysis revealed that 28.1 ± 3.6% and 23.3 ± 2.0% of ARC *Tac3*-expressing cells exhibited EGFP immunoreactivity in *Ncor2*-shRNA #1- or scrambled-shRNA-treated OVX + high E2 *Kiss1-Cre* rats, respectively. No significant differences were observed in the numbers of *Tac3*-expressing cells ($P = 0.8627$) and *Tac3*/EGFP-double-positive cells ($P = 0.4081$), and the percentages of *Tac3*-positive cells co-expressing EGFP ($P = 0.3712$) between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups in OVX + high E2 *Kiss1-Cre* rats (Fig. 4C). Representative images of *Kiss1*-expressing cells in the anterior and posterior ARC of OVX + high E2 *Kiss1-Cre* virgin rats transfected with *Ncor2*-targeting shRNA #1 or scrambled-shRNA are shown in Fig. 4D. Quantitative analysis revealed that the number of ARC *Kiss1*-expressing cells was significantly higher in *Kiss1-Cre* rats transfected with *Ncor2*-shRNA #1 compared to *Kiss1-Cre* control rats transfected with scrambled-shRNA (Fig. 4E; $P = 0.0276$). Moreover, the intensity of *Kiss1* mRNA signals was significantly higher in the *Ncor2*-shRNA #1 group than in the control group (Fig. 4E; $P = 0.0235$).

Next, we examined the effect of ARC *Kiss1*-dependent *Ncor2* knockdown on ARC *Kiss1* expression in OVX + low E2 *Kiss1-Cre* lactating rats (Fig. 5A). Representative images of *Tac3*/EGFP-double-positive cells in the anterior and posterior ARC of OVX + low E2 *Kiss1-Cre* lactating rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA are shown in Fig. 5B. Quantitative analysis revealed that 35.4 ± 3.5% and 27.0 ± 5.3% of *Tac3*-expressing cells exhibited EGFP immunoreactivity in *Ncor2*-shRNA- or scrambled-shRNA-treated

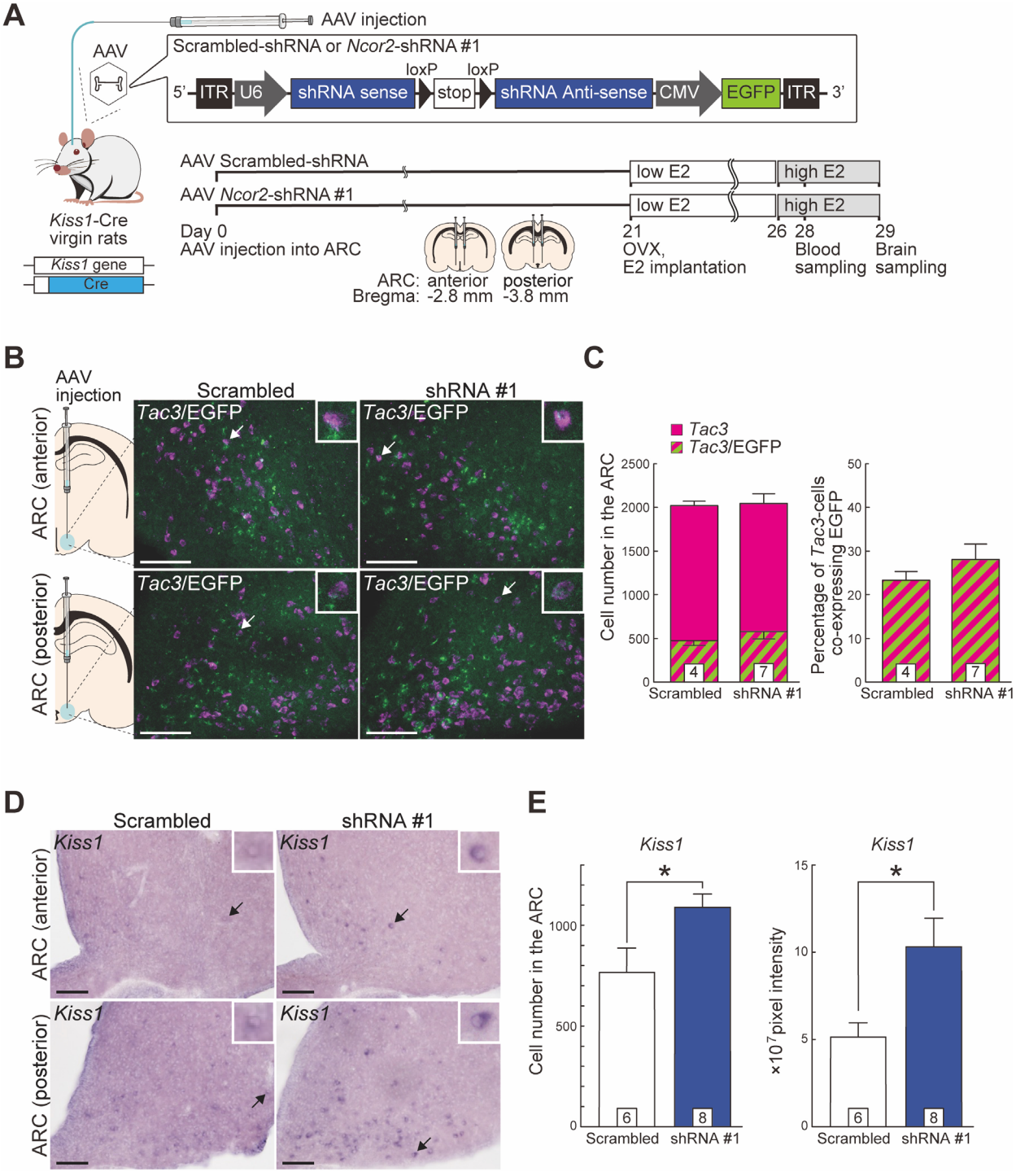


Fig. 4. Effects of *Ncor2*-shRNA administration into the ARC on *Kiss1* repression in the ARC of OVX + high E2 *Kiss1-Cre* virgin rats. (A) Schematic of the procedure for *Ncor2* knockdown in ARC *Kiss1*-expressing cells in OVX + high E2 *Kiss1-Cre* virgin rats. An adeno-associated virus (AAV) vector carrying Cre-dependent *Ncor2*-shRNA #1, corresponding to siRNA #1, or scrambled-shRNA (control) with a Cre-independent CMV promoter-driven EGFP reporter was bilaterally administered into the anterior and posterior ARC of *Kiss1-Cre* virgin rats. Animals were OVX three weeks after AAV administration and treated with low E2 for five days and high E2 for two days. Blood and brain samples were collected under the high E2 condition. (B) *Tac3* (a marker for ARC kisspeptin neurons)-expressing cells detected by ISH (magenta) and EGFP protein detected by immunohistochemistry (green) in the anterior and posterior ARC of representative OVX + high E2 *Kiss1-Cre* virgin rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA. (C) The numbers of ARC *Tac3*-expressing cells (magenta column) and *Tac3*/EGFP-double-positive cells (magenta and green striped column), and the percentages of *Tac3*-positive cells co-expressing EGFP in the *Ncor2*-shRNA- or scrambled-shRNA-treated groups. The numbers of *Tac3*-expressing cells and *Tac3*/EGFP-double-positive cells, and the percentages were comparable between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups. (D) *Kiss1*-expressing cells detected by ISH in the ARC of representative OVX + high E2 *Kiss1-Cre* virgin rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA. (E) The number of ARC *Kiss1*-expressing cells in OVX + high E2 *Kiss1-Cre* virgin rats treated with *Ncor2*-shRNA #1 was significantly higher than that in scrambled-shRNA-treated controls (* $P < 0.05$, Student's *t*-test). The intensity of ARC *Kiss1* mRNA signals in OVX + high E2 *Kiss1-Cre* virgin rats treated with *Ncor2*-shRNA #1 was significantly higher than that in scrambled-shRNA-treated controls (* $P < 0.05$, Student's *t*-test). The insets indicate representative *Tac3*/EGFP-double-positive cells and *Kiss1*-expressing cells (arrows) at higher magnification. Scale bars, 100 μm. The number in each column indicates the number of animals used.

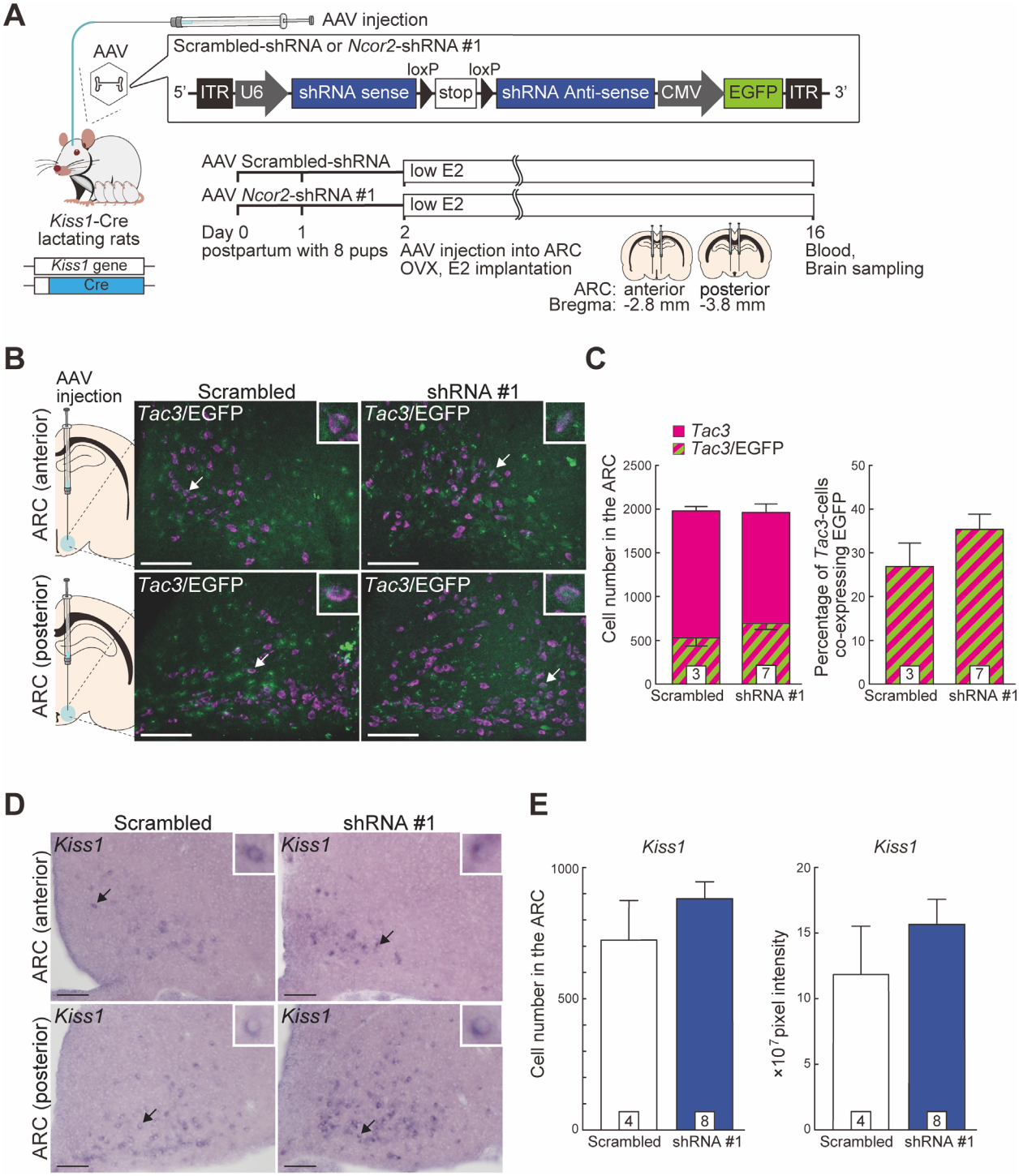


Fig. 5. The effects of *Ncor2*-shRNA administration into the ARC on *Kiss1* mRNA expression in OVX + low E2 *Kiss1-Cre* lactating rats. (A) Schematic of the procedure for *Ncor2* knockdown in ARC *Kiss1*-expressing cells in OVX + low E2 *Kiss1-Cre* lactating rats. The day of delivery was designated as day 0 postpartum, and the number of pups was adjusted to eight on day 1 postpartum. On day 2 postpartum, *Kiss1-Cre* lactating rats were OVX and implanted with low E2, and AAV carrying Cre-dependent *Ncor2*-shRNA #1 or scrambled-shRNA and a Cre-independent CMV promoter-driven EGFP reporter was bilaterally administered into the anterior and posterior ARC. Blood and brain samples were collected on day 16 postpartum. (B) *Tac3*-expressing cells (magenta) and EGFP-immunopositive cells (green) in the anterior and posterior ARC of representative OVX + low E2 *Kiss1-Cre* lactating rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA. (C) The numbers of *Tac3*-expressing cells (magenta column) and *Tac3*/EGFP-double-positive cells (magenta and green striped column), and the percentages of *Tac3*-positive cells co-expressing EGFP in the *Ncor2*-shRNA- or scrambled-shRNA-treated groups. The numbers of *Tac3*-expressing cells and *Tac3*/EGFP-double-positive cells, and the percentages were comparable between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups. (D) *Kiss1*-expressing cells in the ARC of representative OVX + low E2 *Kiss1-Cre* lactating rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA. (E) The number of ARC *Kiss1*-expressing cells in OVX + low E2 *Kiss1-Cre* lactating rats was comparable between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups. The intensity of ARC *Kiss1* mRNA signals in OVX + low E2 *Kiss1-Cre* lactating rats was comparable between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups. The insets indicate representative *Tac3*/EGFP-double-positive cells and *Kiss1*-expressing cells (arrows) at higher magnification. Scale bars, 100 μm. The number in each column indicates the number of animals used.

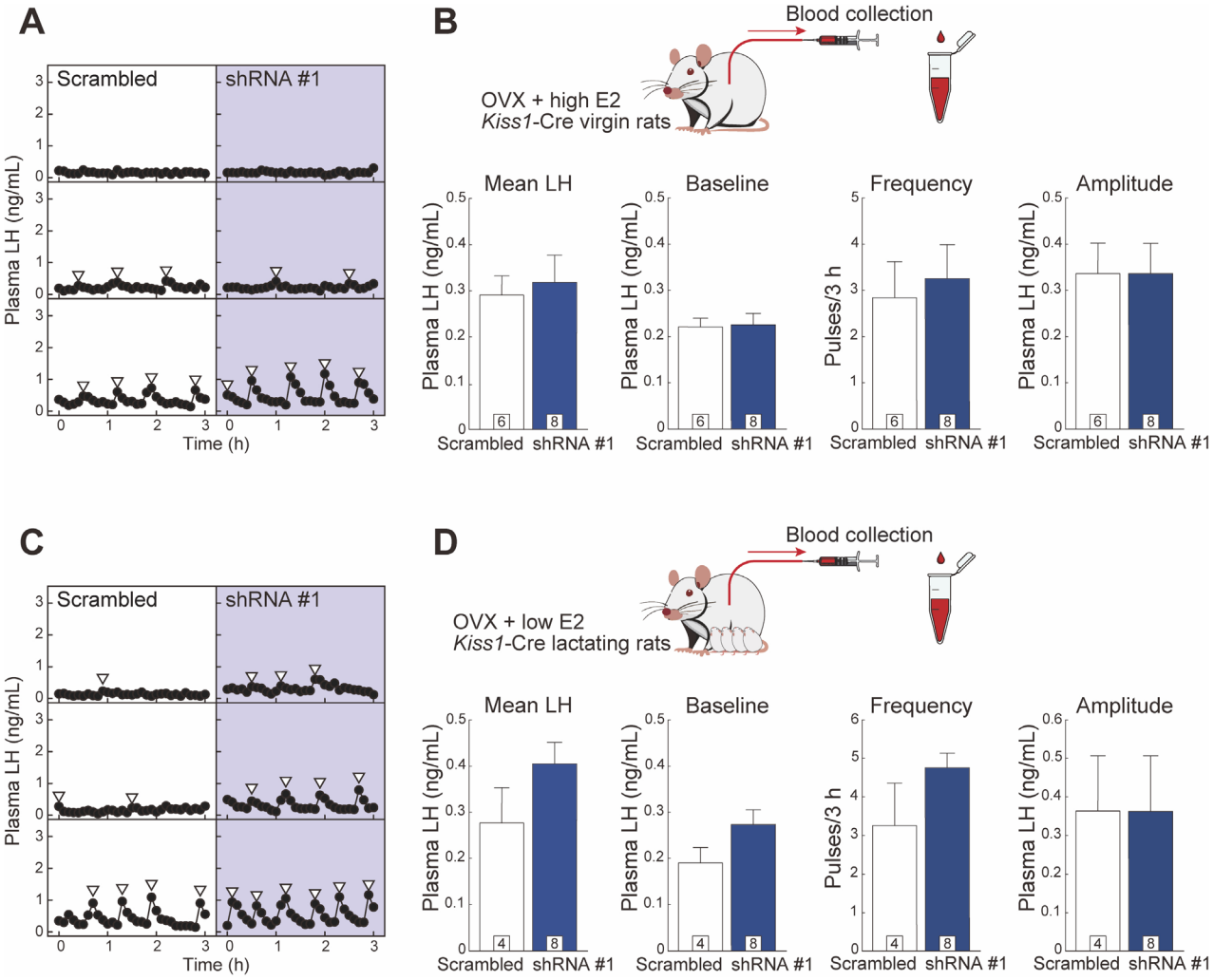


Fig. 6. Administration of *Ncor2*-shRNA into the ARC failed to affect pulsatile luteinizing hormone (LH) release in both OVX + high E2 *Kiss1-Cre* virgin rats and OVX + low E2 *Kiss1-Cre* lactating rats. (A) Plasma LH profiles in OVX + high E2 *Kiss1-Cre* rats administered with AAV carrying *Ncor2*-shRNA #1 or scrambled-shRNA (control) into the bilateral anterior and posterior ARC as described in Figs. 4A and 5A. Arrowheads indicate the peaks of LH pulses identified by the PULSAR computer program. (B) The mean plasma LH concentrations, as well as the baseline, frequency, and amplitude of LH pulses, were comparable between the groups ($P > 0.7$, Student's *t*-test). (C) Plasma LH profiles in OVX + low E2 *Kiss1-Cre* lactating rats injected with *Ncor2*-shRNA #1 or scrambled-shRNA into the ARC. (D) The mean LH concentrations ($P = 0.1626$), as well as the baseline ($P = 0.1387$) and frequency ($P = 0.1316$) of LH pulses, tended to be higher in the *Ncor2*-shRNA #1-transfected OVX + low E2 lactating rats than in scrambled-shRNA-treated lactating rats (Student's *t*-test). The numbers in each column indicate the number of animals used.

OVX + low E2 *Kiss1-Cre* lactating rats, respectively. No significant differences were observed in the numbers of *Tac3*-expressing cells ($P = 0.8890$) and *Tac3*/EGFP-double-positive cells ($P = 0.2261$), and the percentages of *Tac3*-positive cells co-expressing EGFP ($P = 0.2242$) between the *Ncor2*-shRNA #1- and scrambled-shRNA-treated groups in OVX + low E2 *Kiss1-Cre* lactating rats (Fig. 5C). A few *Kiss1*-expressing cells were found in the ARC of OVX + low E2 *Kiss1-Cre* lactating rats transfected with either *Ncor2*-shRNA #1 or scrambled-shRNA (Fig. 5D). The number of *Kiss1*-expressing cells ($P = 0.3025$) and the intensity of *Kiss1* mRNA signals ($P = 0.3221$) tended to be higher in OVX + low E2 *Kiss1-Cre* lactating rats transfected with *Ncor2*-shRNA #1 compared to scrambled-shRNA-treated rats (Fig. 5E).

Ncor2 knockdown failed to block estrogen-induced LH pulse suppression in female rats

Representative plasma LH profiles of OVX + high E2 *Kiss1-Cre* rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA are shown in Fig. 6A. Some rats in both groups exhibited suppressed LH pulses, while others displayed clear LH pulses. The mean plasma LH concentrations were comparable between OVX + high E2 *Kiss1-Cre* rats treated with *Ncor2*-shRNA #1 and those treated with scrambled-shRNA ($P = 0.7343$). Similarly, no significant differences were observed in the baseline ($P = 0.8877$), frequency ($P = 0.7130$), or amplitude ($P = 0.8573$) of LH pulses between the two groups (Fig. 6B). Figure 6C presents plasma LH profiles from representative OVX + low E2 *Kiss1-Cre* lactating rats treated with *Ncor2*-shRNA #1 or scrambled-shRNA. The mean LH concentrations ($P = 0.1626$), and the baseline ($P = 0.1387$) and frequency ($P = 0.1316$) of LH pulses tended to be higher in *Ncor2*-shRNA #1-transfected OVX + low E2 lactating rats compared to scrambled-shRNA-treated lactating rats (Fig. 6D).

Discussion

This study demonstrated that NCOR2, an ERα corepressor, partly mediates the repression of ARC *Kiss1* expression induced by proestrous levels of estrogen in virgin female rats. This is because the majority (> 80%) of ARC kisspeptin neurons expressed *Ncor2*, and *Kiss1*-dependent *Ncor2* knockdown significantly increased both the number of ARC *Kiss1*-expressing cells and the intensity of *Kiss1* mRNA signals in OVX + high E2 *Kiss1-Cre* virgin rats. NCOR2, which functions as an adaptor to recruit HDAC, can bind to ERα in an estrogen-dependent manner to repress target genes by inducing histone deacetylation [54]. This notion is consistent with previous studies showing that high (proestrous) levels of E2 profoundly repressed ARC *Kiss1* mRNA expression in OVX rats and mice [3, 12, 20, 31, 55] and significantly decreased histone acetylation at the *Kiss1* promoter region in the ARC of mice [31]. Therefore, the current ARC *Kiss1*-dependent *Ncor2* silencing likely prevented estrogen-induced histone deacetylation at the ARC *Kiss1* promoter, thereby partially blocking high E2-induced *Kiss1* repression in ARC kisspeptin neurons in female rats.

This study, however, failed to evaluate the role of NCOR2 in low E2-induced repression of ARC *Kiss1* expression in lactating rats because *Kiss1*-dependent *Ncor2* knockdown failed to fully restore the number of ARC *Kiss1*-expressing cells or the intensity of *Kiss1* mRNA signals in OVX + low E2 *Kiss1-Cre* lactating rats during late lactation. Notably, our previous study demonstrated that central antagonism of type-2 somatostatin receptor (SSTR2) signaling significantly increased the number of ARC *Kiss1*-expressing cells in OVX lactating rats treated with low E2 during late lactation [56]. This suggests that inhibitory inputs, such as somatostatin-SSTR2 signaling, may partly contribute to the low E2-induced repression of ARC *Kiss1* expression in lactating rats. Moreover, conditional knockout of the prolactin receptor (PRLR) in neurons expressing calcium/calmodulin-dependent protein kinase IIα, which is highly expressed in forebrain neurons, increased kisspeptin immunoreactivity in the ARC of lactating mice [57]. This implies that PRLR-mediated inhibitory regulation may contribute to repressing kisspeptin expression in mice during lactation. Alternatively, other intracellular ERα-mediated repressive cofactors, such as DAX1 and SP3, may play a role in the lactational repression of ARC *Kiss1* expression, because DAX1 was reported to repress *in vivo Kiss1* expression in the mouse hypothalamus [58], and SP3 was shown to repress *in vitro Kiss1* expression in HEK293T cells [59]. Further studies are necessary to clarify the roles of NCOR2 and/or other ERα corepressors in low E2-induced ARC *Kiss1* repression during lactation.

The present study demonstrated that *Kiss1*-dependent *Ncor2* knockdown failed to restore LH pulses in OVX + high E2 *Kiss1-Cre* rats, despite a significant increase in the number of ARC *Kiss1*-expressing cells and the intensity of *Kiss1* mRNA signals in *Ncor2*-shRNA #1-treated OVX + high E2 virgin rats compared to scrambled-shRNA-treated OVX + high E2 *Kiss1-Cre* control rats. This inconsistency between ARC *Kiss1* expression and LH pulses may partly result from inefficient *Ncor2* knockdown in ARC kisspeptin neurons. Alternatively, NCOR2 may be involved in the repression of high E2-induced ARC *Kiss1* expression, but not in kisspeptin release. Furthermore, the current *Kiss1*-dependent *Ncor2* knockdown failed to significantly increase the number of ARC *Kiss1*-expressing cells, the intensity of *Kiss1* mRNA signals, or LH pulses in OVX + low E2 *Kiss1-Cre* lactating rats. Previous studies revealed that low E2 treatment enhanced paraventricular nucleus (PVN) dynorphin A (Dyn) expression in OVX virgin rats, and PVN Dyn neurons mediated lactational or glucoprivic LH pulse suppression in female rats [35, 60]. Thus, the repression of *Kiss1* mRNA expression and kisspeptin release may be regulated by distinct mechanisms under negative feedback levels of estrogen in female rats.

In conclusion, this study demonstrated that NCOR2 in ARC kisspeptin neurons partly mediates the repression of ARC *Kiss1* expression in the presence of proestrous levels of estrogen in female rats. On the other hand, the role of NCOR2 in low E2-induced ARC *Kiss1* repression in lactating rats could not be evaluated. Further studies are required to investigate the role of NCOR2 and/or other intracellular factors involved in the repression of ARC *Kiss1* expression in lactating rats under diestrous levels of estrogen.

Conflict of interests: The authors declare that they have no competing interests.

Acknowledgments

The authors thank the National Hormone and Peptide Program for providing rat LH-RIA kits and Drs. G.R. Merriam and K.W. Wachter for the PULSAR computer program. Radioimmunoassays were performed at the Radioisotope Research Center, Nagoya University, Japan. This work was supported by the Japan Society for the Promotion of Science KAKENHI grant numbers JP21H05031, JP21K19186 (to H. Tsukamura), JP23H02362, JP23K27055 (to N.I.), and JP20H03127, JP22K19245, JP24K01905 (to Y.U.); the Graduate Program of Transformative Chem-Bio Research in Nagoya University, supported by MEXT (WISE Program) (to H. Tsuchida and M.N.); and "Nagoya University Interdisciplinary Frontier Fellowship" supported by Nagoya University and the Japan Science and Technology Agency, the establishment of university fellowships towards the creation of science technology innovation, grant number JPMJFS2120 (to M.T.).

References

1. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA.** A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 2004; **145**: 4073–4077. [Medline] [CrossRef]
2. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA.** Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of *KiSS-1* mRNA in the male rat. *Neuroendocrinology* 2004; **80**: 264–272. [Medline] [CrossRef]
3. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K.** Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology* 2005; **146**: 4431–4436. [Medline] [CrossRef]
4. **Uenoyama Y, Nakamura S, Hayakawa Y, Ikegami K, Watanabe Y, Deura C, Minabe S, Tomikawa J, Goto T, Ieda N, Inoue N, Sanbo M, Tamura C, Hirabayashi M, Maeda KI, Tsukamura H.** Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in *Kiss1* knockout rats. *J Neuroendocrinol* 2015; **27**: 187–197. [Medline] [CrossRef]
5. **Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MBL, Colledge WH, Caraty A, Aparicio SA.** Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA* 2005; **102**: 1761–1766. [Medline] [CrossRef]
6. **Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda K-I, Okamura H.** Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* 2009; **21**: 813–821. [Medline] [CrossRef]
7. **Naniwa Y, Nakatsukasa K, Setsuda S, Oishi S, Fujii N, Matsuda F, Uenoyama Y, Tsukamura H, Maeda K, Ohkura S.** Effects of full-length kisspeptin administration on follicular development in Japanese Black beef cows. *J Reprod Dev* 2013; **59**: 588–594. [Medline] [CrossRef]
8. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM.** Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci USA* 2005; **102**: 2129–2134. [Medline] [CrossRef]

9. **Guerriero KA, Keen KL, Millar RP, Terasawa E.** Developmental changes in GnRH release in response to kisspeptin agonist and antagonist in female rhesus monkeys (Macaca mulatta): implication for the mechanism of puberty. *Endocrinology* 2012; **153**: 825–836. [Medline] [CrossRef]
10. **Kalil B, Ramaswamy S, Plant TM.** The distribution of substance P and kisspeptin in the mediobasal hypothalamus of the male rhesus monkey and a comparison of intravenous administration of these peptides to release GnRH as reflected by LH secretion. *Neuroendocrinology* 2016; **103**: 711–723. [Medline] [CrossRef]
11. **Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR.** Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab* 2005; **90**: 6609–6615. [Medline] [CrossRef]
12. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K.** Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* 2007; **53**: 367–378. [Medline] [CrossRef]
13. **Clarkson J, Herbison AE.** Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 2006; **147**: 5817–5825. [Medline] [CrossRef]
14. **Herbison AE.** A simple model of estrous cycle negative and positive feedback regulation of GnRH secretion. *Front Neuroendocrinol* 2020; **57**: 100837. [Medline] [CrossRef]
15. **Tsukamura H.** Kobayashi Award 2019: The neuroendocrine regulation of the mammalian reproduction. *Gen Comp Endocrinol* 2022; **315**: 113755. [Medline] [CrossRef]
16. **Goodman RL, Herbison AE, Lehman MN, Navarro VM.** Neuroendocrine control of gonadotropin-releasing hormone: Pulsatile and surge modes of secretion. *J Neuroendocrinol* 2022; **34**: e13094. [Medline] [CrossRef]
17. **Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y, Takase K, Inoue N, Ohkura S, Uenoyama Y, Maeda K, Tsukamura H.** Molecular characterization and estrogen regulation of hypothalamic *KISS1* gene in the pig. *Biol Reprod* 2010; **82**: 313–319. [Medline] [CrossRef]
18. **Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H.** Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* 2009; **21**: 813–821. [Medline] [CrossRef]
19. **Hassaneen A, Naniwa Y, Suetomi Y, Matsuyama S, Kimura K, Ieda N, Inoue N, Uenoyama Y, Tsukamura H, Maeda K-I, Matsuda F, Ohkura S.** Immunohistochemical characterization of the arcuate kisspeptin/neurokinin B/dynorphin (KNDy) and preoptic kisspeptin neuronal populations in the hypothalamus during the estrous cycle in heifers. *J Reprod Dev* 2016; **62**: 471–477. [Medline] [CrossRef]
20. **Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA.** Regulation of *Kiss1* gene expression in the brain of the female mouse. *Endocrinology* 2005; **146**: 3686–3692. [Medline] [CrossRef]
21. **Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA.** *Kiss1* neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci* 2006; **26**: 6687–6694. [Medline] [CrossRef]
22. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A.** Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* 2006; **401**: 225–230. [Medline] [CrossRef]
23. **Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M.** Sexual differentiation of *Kiss1* gene expression in the brain of the rat. *Endocrinology* 2007; **148**: 1774–1783. [Medline] [CrossRef]
24. **Yamada S, Uenoyama Y, Deura C, Minabe S, Naniwa Y, Iwata K, Kawata M, Maeda K-I, Tsukamura H.** Oestrogen-dependent suppression of pulsatile luteinising hormone secretion and *kiss1* mRNA expression in the arcuate nucleus during late lactation in rats. *J Neuroendocrinol* 2012; **24**: 1234–1242. [Medline] [CrossRef]
25. **Uenoyama Y, Inoue N, Nakamura S, Tsukamura H.** Kisspeptin neurons and estrogen-estrogen receptor α signaling: Unraveling the mystery of steroid feedback system regulating mammalian reproduction. *Int J Mol Sci* 2021; **22**: 9229. [Medline] [CrossRef]
26. **Watanabe Y, Uenoyama Y, Suzuki J, Takase K, Suetomi Y, Ohkura S, Inoue N, Maeda K-I, Tsukamura H.** Oestrogen-induced activation of preoptic kisspeptin neurones may be involved in the luteinising hormone surge in male and female Japanese monkeys. *J Neuroendocrinol* 2014; **26**: 909–917. [Medline] [CrossRef]
27. **Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE.** Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* 2008; **28**: 8691–8697. [Medline] [CrossRef]
28. **Green KA, Carroll JS.** Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. *Nat Rev Cancer* 2007; **7**: 713–722. [Medline] [CrossRef]
29. **Klinge CM.** Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 2000; **65**: 227–251. [Medline] [CrossRef]
30. **Assadullah IN, Ieda N, Kawai N, Ishii H, Ihara K, Inoue N, Uenoyama Y, Tsukamura H.** Co-expression of the calcitonin receptor gene in the hypothalamic kisspeptin neurons in female rats. *Reprod Med Biol* 2018; **17**: 164–172. [Medline] [CrossRef]
31. **Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, Inoue N, Sanbo M, Tomita K, Hirabayashi M, Tanaka S, Imamura T, Okamura H, Maeda K, Tsukamura H.** Epigenetic regulation of *Kiss1* gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc Natl Acad Sci USA* 2012; **109**: E1294–E1301. [Medline] [CrossRef]
32. **Yamada K, Nagae M, Mano T, Tsuchida H, Hazim S, Goto T, Sanbo M, Hirabayashi M, Inoue N, Uenoyama Y, Tsukamura H.** Sex difference in developmental changes in visualized *Kiss1* neurons in newly generated *Kiss1*-Cre rats. *J Reprod Dev* 2023; **69**: 227–238. [Medline] [CrossRef]
33. **Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K.** Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. *J Endocrinol* 1991; **129**: 321–328. [Medline] [CrossRef]
34. **Horihata K, Inoue N, Uenoyama Y, Maeda KI, Tsukamura H.** Retinoblastoma binding protein 7 is involved in *Kiss1* mRNA upregulation in rodents. *J Reprod Dev* 2020; **66**: 125–133. [Medline] [CrossRef]
35. **Tsuchida H, Mostari P, Yamada K, Miyazaki S, Enomoto Y, Inoue N, Uenoyama Y, Tsukamura H.** Paraventricular dynorphin A neurons mediate LH pulse suppression induced by hindbrain glucoprivation in female rats. *Endocrinology* 2020; **161**: 1–18. [Medline] [CrossRef]
36. **Tsuchida H, Kawai N, Yamada K, Takizawa M, Inoue N, Uenoyama Y, Tsukamura H.** Central μ-opioid receptor antagonism blocks glucoprivic LH pulse suppression and gluconeogenesis/feeding in female rats. *Endocrinology* 2021; **162**: 1–12. [Medline] [CrossRef]
37. **Yamada S, Uenoyama Y, Kinoshita M, Iwata K, Takase K, Matsui H, Adachi S, Inoue K, Maeda K-I, Tsukamura H.** Inhibition of metastin (kisspeptin-54)-GPR54 signaling in the arcuate nucleus-median eminence region during lactation in rats. *Endocrinology* 2007; **148**: 2226–2232. [Medline] [CrossRef]
38. **Paxinos G, Watson C.** The Rat Brain in Stereotaxic Coordinates: 6th ed. Elsevier; 2006.
39. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA.** Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* 2009; **29**: 11859–11866. [Medline] [CrossRef]
40. **Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H.** Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci* 2010; **30**: 3124–3132. [Medline] [CrossRef]
41. **True C, Kirigiti M, Ciofi P, Grove KL, Smith MS.** Characterisation of arcuate nucleus kisspeptin/neurokinin B neuronal projections and regulation during lactation in the rat. *J Neuroendocrinol* 2011; **23**: 52–64. [Medline] [CrossRef]
42. **Sugimoto A, Tsuchida H, Ieda N, Ikegami K, Inoue N, Uenoyama Y, Tsukamura H.** Somatostatin-somatostatin receptor 2 signaling mediates LH pulse suppression in lactating rats. *Endocrinology* 2019; **160**: 473–483. [Medline] [CrossRef]
43. **Nagae M, Uenoyama Y, Okamoto S, Tsuchida H, Ikegami K, Goto T, Majarune S, Nakamura S, Sanbo M, Hirabayashi M, Kobayashi K, Inoue N, Tsukamura H.** Direct evidence that KNDy neurons maintain gonadotropin pulses and folliculogenesis as the GnRH pulse generator. *Proc Natl Acad Sci USA* 2021; **118**: 1–11. [Medline] [CrossRef]
44. **Sharma N, Pollina EA, Nagy MA, Yap E-L, DiBiase FA, Hrvatin S, Hu L, Lin C, Greenberg ME.** ARNT2 tunes activity-dependent gene expression through NCoR2-mediated repression and NPAS4-mediated activation. *Neuron* 2019; **102**: 390–406.e9. [Medline] [CrossRef]
45. **Iemolo A, Montilla-Perez P, Lai I-C, Meng Y, Nolan S, Wen J, Rusu I, Dulcis D, Telese F.** A cell type-specific expression map of NCoR1 and SMRT transcriptional co-repressors in the mouse brain. *J Comp Neurol* 2020; **528**: 2218–2238. [Medline] [CrossRef]
46. **Suzuki T, Morimoto N, Akaike A, Osakada F.** Multiplex neural circuit tracing with G-deleted rabies viral vectors. *Front Neural Circuits* 2020; **13**: 77. [Medline] [CrossRef]
47. **Hitz C, Wurst W, Kühn R.** Conditional brain-specific knockdown of MAPK using Cre/loxP regulated RNA interference. *Nucleic Acids Res* 2007; **35**: e90. [Medline] [CrossRef]
48. **Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS.** Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002; **16**: 948–958. [Medline] [CrossRef]
49. **Zharikov AD, Cannon JR, Tapias V, Bai Q, Horowitz MP, Shah V, El Ayadi A, Hastings TG, Greenamyre JT, Burton EA.** shRNA targeting α-synuclein prevents neurodegeneration in a Parkinson's disease model. *J Clin Invest* 2015; **125**: 2721–2735. [Medline] [CrossRef]
50. **Tsuchida H, Takizawa M, Nonogaki M, Inoue N, Uenoyama Y, Tsukamura H.** Enkephalin-δ opioid receptor signaling partly mediates suppression of LH release during early lactation in rats. *J Reprod Dev* 2023; **69**: 192–197. [Medline] [CrossRef]
51. **Uenoyama Y, Nonogaki M, Tsuchida H, Takizawa M, Matsuzaki S, Inoue N, Tsukamura H.** Central δ/κ opioid receptor signaling pathways mediate chronic and/or acute suckling-induced LH suppression in rats during late lactation. *J Reprod Dev* 2024; **70**: 327–337. [Medline] [CrossRef]
52. **Hazim S, Seki S, Yabushita R, Nagae M, Tsuchida H, Hirabayashi M, Uenoyama Y, Tsukamura H, Inoue N.** AVPV *Kiss1* neuron-specific knockdown of purinergic P2X2 receptor suppresses LH surge and ovulation in *Kiss1-Cre* rats. *J Reprod Dev* 2024; **70**: 379–388. [Medline] [CrossRef]
53. **Merriam GR, Wachter KW.** Algorithms for the study of episodic hormone secretion. *Am J Physiol* 1982; **243**: E310–E318. [Medline]
54. **Suzuki A, Sanda N, Miyawaki Y, Fujimori Y, Yamada T, Takagi A, Murate T, Saito H, Kojima T.** Down-regulation of PROS1 gene expression by 17β-estradiol via estrogen receptor α (ERalpha)-Sp1 interaction recruiting receptor-interacting protein 140 and the corepressor-HDAC3 complex. *J Biol Chem* 2010; **285**: 13444–13453. [Medline] [Cross-

Ref]

55. **Goto T, Tomikawa J, Ikegami K, Minabe S, Abe H, Fukanuma T, Imamura T, Takase K, Sanbo M, Tomita K, Hirabayashi M, Maeda K, Tsukamura H, Uenoyama Y.** Identification of hypothalamic arcuate nucleus-specific enhancer region of *Kiss1* gene in mice. *Mol Endocrinol* 2015; **29**: 121–129. [Medline] [CrossRef]
56. **Sugimoto A, Tsuchida H, Nagae M, Inoue N, Uenoyama Y, Tsukamura H.** Central somatostatin-somatostatin receptor 2 signaling mediates lactational suppression of luteinizing hormone release via the inhibition of glutamatergic interneurons during late lactation in rats. *J Reprod Dev* 2022; **68**: 190–197. [Medline] [CrossRef]
57. **Hackwell ECR, Ladyman SR, Clarkson J, McQuillan HJ, Boehm U, Herbison AE, Brown RSE, Grattan DR.** Prolactin-mediates a lactation-induced suppression of arcuate kisspeptin neuronal activity necessary for lactational infertility in mice. *eLife* 2024; **13**: RP94570.
58. **Ramos-Pittol JM, Fernandes-Freitas I, Milona A, Manchishi SM, Rainbow K, Lam BYH, Tadross JA, Beucher A, Colledge WH, Cebola I, Murphy KG, Miguel-Aliaga I, Yeo GSH, Dhillo WS, Owen BM.** *Dax1* modulates ERα-dependent hypothalamic estrogen sensing in female mice. *Nat Commun* 2023; **14**: 3076. [Medline] [CrossRef]
59. **Li D, Mitchell D, Luo J, Yi Z, Cho S-G, Guo J, Li X, Ning G, Wu X, Liu M.** Estrogen regulates *KiSS1* gene expression through estrogen receptor α and SP protein complexes. *Endocrinology* 2007; **148**: 4821–4828. [Medline] [CrossRef]
60. **Tsuchida H, Nonogaki M, Inoue N, Uenoyama Y, Tsukamura H.** Dynorphin-κ-opioid receptor signaling, but not μ-opioid receptor signaling, partly mediates the suppression of luteinizing hormone release during late lactation in rats. *Neurosci Lett* 2022; **791**: 136920. [Medline] [CrossRef]